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Title:

Nontypeable Haemophilus Influenzae Virulence Factors

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NONTYPEABLE *HAEMOPHILUS INFLUENZAE* VIRULENCE FACTORS

Related Applications

The present application claims priority benefit from U.S. Provisional Application 60/458,234 filed March 27, 2003 which is incorporated herein by reference in its entirety.

Field of Invention

The invention relates to a mutation within the *sap* operon of an avirulent clone of a nontypeable strain of *Haemophilus influenzae* (NTHi). The invention relates to methods of modulating NTHi virulence and NTHi sensitivity to antimicrobial agents. The invention also relates to a novel 110 kDa NTHi outer membrane protein and the polynucleotide that encodes this outer membrane protein. Methods of screening for NTHi infection, and treating and preventing NTHi related disorders are also contemplated.

Background

Otitis media (OM) is a highly prevalent pediatric disease worldwide and is the primary cause for emergency room visits by children (Infante-Rivand and Fernandez, *Epidemiol. Rev.,* 15: 444-465,1993). Recent statistics indicate that 24.5 million physician office visits were made for OM in 1990, representing a greater than 200% increase over those reported in the 1980's. While rarely associated with mortality any longer, the morbidity associated with OM is significant. Hearing loss is a common problem associated with this disease, often times affecting a child's behavior, education and development of language skills (Baldwin, *Am. J. Otol.,* 14: 601-604, 1993; Hunter *et al., Ann. Otol. Rhinol. Laryngol. Suppl.,*163: 59-61, 1994; Teele *et al., J. Infect. Dis.*, 162:685-694, 1990). The socioeconomic impact of OM is also great, with direct and indirect costs of diagnosing and managing OM exceeding $5 billion annually in the U.S. alone (Kaplan *et al., Pediatr. Infect. Dis. J.,* 16: S9-11, 1997).

Whereas antibiotic therapy is common and the surgical placement of tympanostomy tubes has been successful in terms of draining effusions, clearing infection and relieving pain associated with the accumulation of fluids in the middle ear, the emergence of multiple antibiotic-resistant bacteria and the invasive nature associated with tube placement, has illuminated the need for more effective and

accepted approaches to the management and preferably, the prevention of OM. Surgical management of chronic OM involves the insertion of tympanostomy tubes through the tympanic membrane while a child is under general anesthesia. While this procedure is commonplace (prevalence rates are ~ 13%; Bright *et al., Am. J. Public Health*, 83(7): 1026-8, 1993) and is highly effective in terms of relieving painful symptoms by draining the middle ear of accumulated fluids, it too has met with criticism due to the invasive nature of the procedure and its incumbent risks (Berman *et al., Pediatrics*, 93(3):353-63, 1994; Bright *et al., supra.;* Cimons, *ASM News*, 60: 527-528; Paap, *Ann. Pharmacother.*, 30(11): 1291-7, 1996).

Progress in vaccine development is most advanced for *Streptococcus pneumoniae*, the primary causative agent of acute OM (AOM), as evidenced by the recent approval and release of a seven-valent capsular-conjugate vaccine, PREVNAR® (Eskola and Kilpi, *Pedriatr. Infect. Dis. J.* 16: S72-78, 2000). While PREVNAR® has been highly efficacious for invasive pneumococcal disease, coverage for OM has been disappointing (6-8%) with reports of an increased number of OM cases due to serotypes not included in the vaccine (Black *et al., Pedriatr. Infect. Dis J.,* 19: 187-195; Eskola *et al., Pedriatr. Infect. Dis J.,* 19: S72-78, 2000; Eskola *et* al., *N. Engl. J. Med.* 344: 403-409, 2001; Snow *et al., Otol. Neurotol.,* 23: 1-2, 2002). Less progress has been made for nontypeable *Haemophilus influenzae* (NTHi), the gram-negative pathogen that predominates in chronic OM with effusion (Klein, *Pedriatr. Infect. Dis J.*, 16: S5-8, 1997; Spinola *et al., J. Infect. Dis.*, 54: 100-109, 1986). Hampering development of effective vaccines against NTHi, has been the incomplete understanding of the pathogenesis of NTHi-induced middle ear disease. Contributing to this delay was a lack of understanding of the dynamic interplay between microbe-expressed virulence factors and the host's immune response as the disease progresses from one of host immunogenic tolerance of a benign nasopharyngeal commensal, to that of an active defensive reaction to an opportunistic invader of the normally sterile middle ear space.

There has been a poor understanding of how NTHi causes OM in children. The identification of putative virulence factors necessary for induction of OM will contribute significantly to the understanding of the host-pathogen interaction and ultimately, the identification of potential vaccine candidates and targets of chemotherapy. There is a tremendous need to develop more effective and accepted

approaches to the management and preferably, the prevention of otitis media. Vaccine development is a very promising and cost effective method to accomplish this goal (Giebank, *Pedriatr. Infect. Dis J.*, 13(11): 1064-8, 1994: Karma *et al., Int. J. Pedritr. Otorhinolaryngol.*, 32(Suppl.): S127-34, 1995).

Summary of Invention

Signature-tagged mutagenesis screening of avirulent NTHi clones using a transbullar chinchilla model of OM identified a mutant that was unable to survive in the environment of the middle ear during OM. This mutant of interest harbored an interruption in the *sapF* gene within the *sap* operon. The mutant is denoted herein as *sapF*::mTn5. This mutant was 3-fold more sensitive to the action of the antimicrobial peptide protamine and displayed a concurrent loss of an approximately 110 kDa outer membrane protein (OMP).

The *sap* operon is known to be involved in conferring resistance to the action of antimicrobial peptides. The *sap* operon was first identified and characterized in *S. typhimurium* where it functions in resistance to the cationic peptide protamine. (Parra-Lopez *et al., EMBO J.* 12: 4053-62, 1993). A search of the available complete and incomplete bacterial genome sequences in NCBI databases revealed *sap* operons in the genomes of *H. influenzae, Pasteurella multocida, Yersinia pestis, S. typhimurium, S. enterica, E. coli, E. chrysanthemi, and V. cholerae.* All of these organisms had the conserved gene order of *sapABCDF* in the operon. The structure of the gene cluster suggests that all *sap* genes were co-transcribed as a single polycistronic mRNA. An interesting finding is the presence of *sapZ,* which encodes a hypothetical transmembrane protein and is unique in *H. influenzae* due to its placement *within* the *sap* operon. In other organisms with a comparable *sap* system, *sapZ* is not co-transcribed with *sapA-F.* The *sapABCDF* gene products are components of an ABC transporter system involved in peptide uptake (Parra-Lopez *et al., supra.*). The SapA protein is a periplasmic dipeptide binding protein. SapB and SapC are transmembrane proteins embedded in the inner membrane. SapD and SapF are two ATP hydrolyzing proteins localized in cytoplasm presumably associated with SapB and SapC. The *sapZ* gene product is an as-yet uncharacterized hypothetical protein that is predicted to be a transmembrane protein with gene homologs in *sap* operon-containing bacteria, *P. multocida, S. typhimurium, S. enterica*, and *E. coli*

0157:H7, and in *Neisseria meningitidis* and *Pseudomonas aeruginosa*, which do not contain a *sap* operon. In bacteria containing the described *sap* system, however, *sapZ* is not located near the *sap* operon in the bacterial genome.

The present invention provides the sequences of the 6 NTHi *sap* genes (*sapA, sapB, sapC, sapD, sapF* and *sapZ*) set out as SEQ ID NOS: 1-6 respectively. The polypeptide gene products encoded by the 6 NTHi *sap* genes (SapA, SapB, SapC, SapD, SapF, and SapZ) are set out as SEQ ID NOS: 7-12 respectively. The polynucleotide sequence of the complete NTHi sap operon is set out as SEQ ID NO: 13.

In vitro phenotypic assays described herein revealed that the *sapF* mutant was more sensitive to the antimicrobial peptide protamine than the parent strain, in addition to its absence of a 110 kDa OMP. This was the first observation about the NTHi *sap* gene playing an essential role in survival in the microenvironment of the chinchilla middle ear and in resistance to an antimicrobial peptide. The invention contemplates identifying the relevant host antimicrobial peptides that may be responsible in part for the rapid clearance of the *sapF* mutant, and determining the identity of the absent OMP, and also the functional linkage between this protein and the SapF protein.

A non-polar in-frame mutation of the NTHi *sap* operon, denoted herein as *sapA::kan*, was more sensitive to chinchilla antimicrobial peptide beta-defensin-1 than the parent strain *in vitro*. This mutation also attenuated bacterial survival *in vivo* in the chinchilla middle ear. These studies further demonstrate that the NTHi *sap* operon is critical to survival *in vivo*.

The present invention also provides for the polynucleotide sequences that encodes a portion of the polypeptide sequence of the novel NTHi 110 kDa OMP protein that is set out as SEQ ID NOS: 21-38. Additional sequence analysis identified the full length sequence of the NTHi 110 kDa OMP set out as SEQ ID NO: 41 that is encoded by the nucleic acid set out in SEQ ID NO: 40.

The present invention also provides for antibodies specific for the NTHi SapA, SapB, SapC, SapD, SapF and SapZ proteins and the NTHi 110 kDa OMP protein of the invention. Methods of detecting NTHi bacteria in a human or in sample, such as serum, sputum, ear fluid, blood, urine, lymphatic fluid and

cerebrospinal fluid are contemplated. These methods include detecting a NTHi *sap* polynucleotides or the NTHi 110 kDa OMP polynucleotide with specific polynucleotide probes or detecting an NTHi Sap protein or the NTHi 110 kDa OMP protein with specific antibodies. The invention also contemplates diagnostic kits which utilize these methods of detecting NTHi bacteria.

According to the present invention, the presence of the functional NTHi Sap proteins and/or the NTHi 110 kDa OMP protein is associated with survivability of the NTHi bacterium within the middle ear. The *sapA* gene has been shown to be upregulated during OM infection of the middle ear in the chinchilla. Expression of SapZ protein as part of the *sap* operon is unique to NTHi and therefore is contemplated to be a target for therapies to infections caused by NTHI. Therefore, the NTHi SapA, SapB, SapC, SapD, SapF, SapZ and 110 kDa OMP protein are contemplated as vaccine candidates and/or targets of chemotherapy. The present invention also contemplates methods of eliciting an immune response to one or more of the NTHi SapA, SapB, SapC, SapD, SapF, SapZ and 110 kDa OMP protein of the invention by administering one or more of those proteins or peptides thereof. In one aspect, these methods involve administering one or more of the NTHi SapA, SapB, SapC, SapD, SapF, SapZ and 110 kDa OMP protein or a peptide thereof as a vaccine for treatment and/or prevention of diseases caused by NTHi infection, such as OM.

As a method of treating or preventing NTHi infection, the present invention contemplates administering a molecule that inhibits expression or the activity of one or more of the NTHi SapA, SapB, SapC, SapD, SapF, SapZ and/or110 kDa OMP proteins. In particular, the invention contemplates methods of treating or preventing NTHi infection comprising modulating expression of one or more of the NTHi SapA, SapB, SapC, SapD, SapF, SapZ and/or 110 kDa OMP protein by administering an antisense oligonucleotide that specifically binds to prevent expression of the appropriate NTHi genes. The invention also contemplates methods of treating or preventing NTHi infection comprising administering antibodies or small molecules that modulate the activity of one or more of the NTHi SapA, SapB, SapC, SapD, SapF, SapZ and 110 kDa OMP protein.

The invention also provides for methods of modulating the virulence of the NTHi bacterium or increasing NTHi sensitivity to antimicrobial agents. These methods include mutating the NTHi genes within the *sap* operon. The *sap* operon is

known to be associated with resistance to antimicrobial agents, and a disruption or mutation within this operon is contemplated to decrease virulence. These method include utilizing methods of intercalating or disrupting the DNA within the *sap* operon.

Polynucleotides and Polypeptides of the Invention

The present invention provides polynucleotide sequences of the NTHi sap operon genes (sapA, sapB, sapC, sapD, sapF and sapZ) set out as SEQ ID NOS: 1-6, respectively. The present invention also provides for the polypeptides encoded by the sap operon polynucleotides of the present invention. In addition, the invention provides for the polynucleotide sequence encoding the NTHi 110 kDa OMP set out in SEQ ID NO: 40. The invention provides for polynucleotides that hybridize under stringent conditions to (a) the complement of the nucleotide sequence of SEQ ID NOS: 1-6, (b) the complement of the nucleotide sequence encoding the SEQ ID NO: 40, (c) a polynucleotide which is an allelic variant of any polynucleotides recited above; (d) a polynucleotide which encodes a species homolog of any of the proteins recited above; or (e) a polynucleotide that encodes a polypeptide comprising a specific domain or truncation of the NTHi polypeptides of the present invention.

The NTHi polynucleotides of the invention also include nucleotide sequences that are substantially equivalent to the polynucleotides recited above. Polynucleotides according to the invention can have, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more typically at least 90%, 91%, 92%, 93%, or 94% and even more typically at least 95%, 96%, 97%, 98% or 99% sequence identity to one of the NTHi *sap* operon polynucleotides or the polynucleotide encoding the NTHi 110 kDa OMP recited above.

Included within the scope of the nucleic acids of the invention are nucleic acid fragments that hybridize under stringent conditions to one of the NTHi *sap* operon polynucleotides of SEQ ID NOS: 1-6 or polynucleotides encoding the NTHi 110 kDa OMP (SEQ ID NO: 40), or complements thereof, which fragment is greater than about 5 nucleotides, preferably 7 nucleotides, more preferably greater than 9 nucleotides and most preferably greater than 17 nucleotides in length. Fragments of, *e.g.*, 15, 17, or 20 nucleotides or more that are selective for (*i.e.*, specifically hybridize to any one of the polynucleotides of the invention) are

contemplated. Probes capable of specifically hybridizing to a polynucleotide can differentiate NTHi polynucleotides of the invention from other polynucleotides in the same family of genes or can differentiate NTHi genes from other bacterial genes, and are preferably based on unique nucleotide sequences.

The term "stringent" is used to refer to conditions that are commonly understood in the art as stringent. Hybridization stringency is principally determined by temperature, ionic strength, and the concentration of denaturing agents such as formamide. Examples of stringent conditions for hybridization and washing are 0.015 M sodium chloride, 0.0015 M sodium citrate at 65-68°C or 0.015 M sodium chloride, 0.0015M sodium citrate, and 50% formamide at 42°C. See Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, (Cold Spring Harbor, N.Y. 1989). More stringent conditions (such as higher temperature, lower ionic strength, higher formamide, or other denaturing agent) may also be used, however, the rate of hybridization will be affected. In instances wherein hybridization of deoxyoligonucleotides is concerned, additional exemplary stringent hybridization conditions include washing in 6x SSC 0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos).

Other agents may be included in the hybridization and washing buffers for the purpose of reducing non-specific and/or background hybridization. Examples are 0.1% bovine serum albumin, 0.1% polyvinyl-pyrrolidone, 0.1% sodium pyrophosphate, 0.1% sodium dodecylsulfate, $NaDodSO_4$, (SDS), ficoll, Denhardt's solution, sonicated salmon sperm DNA (or other non-complementary DNA), and dextran sulfate, although other suitable agents can also be used. The concentration and types of these additives can be changed without substantially affecting the stringency of the hybridization conditions. Hybridization experiments are usually carried out at pH 6.8-7.4, however, at typical ionic strength conditions, the rate of hybridization is nearly independent of pH. *See* Anderson *et al.*, *Nucleic Acid Hybridisation: A Practical Approach*, Ch. 4, IRL Press Limited (Oxford, England). Hybridization conditions can be adjusted by one skilled in the art in order to accommodate these variables and allow DNAs of different sequence relatedness to form hybrids.

The sequences falling within the scope of the present invention are not limited to these specific sequences, but also include allelic and species variations thereof. Allelic and species variations can be routinely determined by comparing the sequence provided in SEQ ID NOS: 1-6, the nucleic acid sequence encoding the NTHi 110 kDa OMP polypeptide (SEQ ID NO: 40), a representative fragment thereof, or a nucleotide sequence at least 90% identical, preferably 95% identical, to SEQ ID NOS: 1-6 or SEQ ID NO: 40, with a sequence from another isolate of the same species. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package, including GAP (Devereux *et al.*, *Nucl. Acid. Res.*, 12: 387, 1984; Genetics Computer Group, University of Wisconsin, Madison, WI), BLASTP, BLASTN, and FASTA (Altschul *et al.*, *J. Mol. Biol.*, 215: 403-410, 1990). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (*BLAST Manual,* Altschul *et al.* NCB/NLM/NIH Bethesda, MD 20894; Altschul *et al., supra*). The well-known Smith Waterman algorithm may also be used to determine identity.

Furthermore, to accommodate codon variability, the invention includes nucleic acid molecules coding for the same amino acid sequences as do the specific open reading frames (ORF) disclosed herein. In other words, in the coding region of an ORF, substitution of one codon for another codon that encodes the same amino acid is expressly contemplated. The present invention further provides isolated NTHi polypeptides encoded by the NTHi nucleic acid fragments of the present invention or by degenerate variants of the nucleic acid fragments of the present invention. The term "degenerate variant" refers to nucleotide fragments that differ from a nucleic acid fragment of the present invention (*e.g.*, an ORF) by nucleotide sequence but, due to the degeneracy of the genetic code, encode an identical NTHi polypeptide sequence. Preferred nucleic acid fragments of the present invention are the ORFs that encode proteins.

The isolated polypeptides of the invention include, but are not limited to, a polypeptide comprising: the amino acids encoded by the nucleotide sequences SEQ ID NOS: 7-12, the nucleotide sequence encoding NTHi 110 kDa OMP (SEQ ID NO: 41), or the corresponding full length or mature protein. Polypeptides of the invention also include polypeptides preferably with biological or immunogenic

activity that are encoded by: (a) a polynucleotide having the nucleotide sequences set forth in SEQ ID NOS: 1-6 or SEQ ID NO: 40 or (b) polynucleotides encoding the amino acid sequence set forth as SEQ ID NOS: 7-12 or (c) a polynucleotide having the nucleotide sequence encoding the amino acid sequences set forth as SEQ ID NO: 41, (d) polynucleotides that hybridize to the complement of the polynucleotides of either (a), (b) or (c) under stringent hybridization conditions.

The invention also provides biologically active or immunogenically active variants of the polypeptides of the present invention; and "substantial equivalents" thereof (*e.g.*, with at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, 86%, 87%, 88%, 89%, at least about 90%, 91%, 92%, 93%, 94%, typically at least about 95%, 96%, 97%, more typically at least about 98%, or most typically at least about 99% amino acid identity) that retain biological and/or immunogenic activity. Polypeptides encoded by allelic variants may have a similar, increased, or decreased activity compared to one of the polypeptides encoded by the polynucleotides comprising SEQ ID NOS: 1-6 or the (NTHi 110 kDa OMP polypeptide (SEQ IS NO: 41).

The invention also provides for NTHi polypeptides with one or more conservative amino acid substitutions that do not affect the biological and/or immunogenic activity of the polypeptide. Alternatively, the NTHi polypeptides of the invention are contemplated to have conservative amino acids substitutions that may or may not alter biological activity. The term "conservative amino acid substitution" refers to a substitution of a native amino acid residue with a nonnative residue, including naturally occurring and nonnaturally occurring amino acids, such that there is little or no effect on the polarity or charge of the amino acid residue at that position. For example, a conservative substitution results from the replacement of a non-polar residue in a polypeptide with any other non-polar residue. Further, any native residue in the polypeptide may also be substituted with alanine, according to the methods of "alanine scanning mutagenesis". Naturally occurring amino acids are characterized based on their side chains as follows: basic: arginine, lysine, histidine; acidic: glutamic acid, aspartic acid; uncharged polar: glutamine, asparagine, serine, threonine, tyrosine; and non-polar: phenylalanine, tryptophan, cysteine, glycine, alanine, valine, proline, methionine, leucine, norleucine, isoleucine General rules for amino acid substitutions are set forth in Table 1 below.

Table 1
Amino Acid Substitutions

Original Residues	Exemplary Substitutions	Preferred Substitutions
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln	Gln
Asp	Glu	Glu
Cys	Ser, Ala	Ser
Gln	Asn	Asn
Glu	Asp	Asn
Gly	Pro, Ala	Ala
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe,	Leu
Leu	Norleucine, Ile, Val, Met,	Leu
Lys	Arg, 1,4 Diaminobutyric	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Arg
Pro	Ala	Gly
Ser	Thr, Ala, Cys	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala,	Leu

Antisense polynucleotides complementary to the polynucleotides encoding one of the NTHi *sap* operon proteins and NTHi 110 kDa OMP protein are also provided.

Antisense technology may be employed to inhibit the activity of NTHi SapA, SapB, SapC, SapD, SapF, SapZ or NTHi 110 kDa OMP protein. Such inhibition may be effected by nucleic acid molecules which are complementary to and hybridize to expression control sequences (triple helix formation) or to *sap* operon mRNA or the 110 kDa OMP mRNA. For example, antisense DNA, RNA or RNAi molecules, which have a sequence that is complementary to at least a portion of the selected gene(s) can be introduced into the cell. Antisense probes may be designed by

available techniques using the nucleotide sequence of NTHi *sap* operon or the gene that encodes the NTHi 110 kDa OMP protein disclosed herein. Typically, each such antisense molecule will be complementary to the start site (5' end) of each selected *sap* operon gene or the gene encoding the 110 kDa OMP protein. When the antisense molecule then hybridizes to the corresponding mRNA, translation of this mRNA is prevented or reduced.

Alternatively, gene therapy may be employed to create a dominant-negative inhibitor of one of the NTHi *sap* operon gene products or the NTHi 110 kDa OMP protein. The DNA encoding a mutant polypeptide of these polypeptides can be prepared and introduced into the cells of a patient using either viral or non-viral methods. Each such mutant is typically designed to compete with endogenous polypeptide in its biological role.

In some cases, it may be desirable to prepare nucleic acid molecules encoding variants of the *sap* operon gene product or the NTHi 110 kDa OMP protein. Nucleic acid molecules encoding variants may be produced using site directed mutagenesis, PCR amplification, or other appropriate methods, where the primer(s) have the desired point mutations (see Sambrook *et al.*, *supra*, and Ausubel *et al.*, *supra*, for descriptions of mutagenesis techniques). Chemical synthesis using methods described by Engels *et al.*, *supra*, may also be used to prepare such variants. Other methods known to the skilled artisan may be used as well.

Homologous recombination may also be used to introduce mutations in genes of interest. The basic technique was developed as a method for introducing specific mutations into specific regions of the mammalian genome (Thomas *et al.*, *Cell*, 44:419-428, 1986; Thomas and Capecchi, *Cell*, 51:503-512, 1987; Doetschman *et al.*, *Proc. Natl. Acad. Sci.*, 85:8583-8587, 1988) or to correct specific mutations within defective genes (Doetschman *et al.*, *Nature*, 330:576-578, 1987). Exemplary homologous recombination techniques are described in U.S. Patent No. 5,272,071.

Through homologous recombination, the DNA sequence to be inserted into the genome can be directed to a specific region of the gene of interest by attaching it to targeting DNA. The targeting DNA is a nucleotide sequence that is complementary (homologous) to a region of the genomic DNA. Small pieces of targeting DNA that are complementary to a specific region of the genome are put in

contact with the parental strand during the DNA replication process. It is a general property of DNA that has been inserted into a cell to hybridize, and therefore, recombine with other pieces of endogenous DNA through shared homologous regions. If this complementary strand is attached to an oligonucleotide that contains a mutation or a different sequence or an additional nucleotide, it too is incorporated into the newly synthesized strand as a result of the recombination. As a result of the proofreading function, it is possible for the new sequence of DNA to serve as the template. Thus, the transferred DNA is incorporated into the genome.

Attached to these pieces of targeting DNA are regions of DNA which may interact with or control the expression of a *sap* operon gene product of the NTHi 110 kDa OMP, *e.g.*, flanking sequences. For example, a promoter/enhancer element, a suppresser, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired NTHi polypeptide. The control element controls a portion of the DNA present in the host cell genome. Thus, the expression of the desired NTHi polypeptide may be achieved not by transfection of DNA that encodes NTHi polypeptide itself, but rather by the use of targeting DNA (containing regions of homology with the endogenous gene of interest) coupled with DNA regulatory segments that provide the endogenous gene sequence with recognizable signals for transcription of an NTHi polypeptide.

The invention contemplates that polynucleotides of the invention may be inserted in a vector for amplification or expression. For expression, the polynucleotides are operatively linked to appropriate expression control sequence such as a promoter and polyadenylation signal sequences. Further provided are cells containing polynucleotides of the invention. Exemplary prokaryotic hosts include bacteria such as *E. coli*, *Bacillus, Streptomyces, Pseudomonas, Salmonella* and *Serratia*.

The term "isolated" refers to a substance removed from, and essentially free of, the other components of the environment in which it naturally exists. For example, a polypeptide is separated from other cellular proteins or a DNA is separated from other DNA flanking it in a genome in which it naturally occurs.

Antibodies and Methods for Eliciting an Immune Response

The invention provides antibodies which bind to antigenic epitopes unique to one of the NTHi SapA, SapB, SapC, SapD, SapF, SapZ and NTHi 110 kDa OMP polypeptides. Also provided are antibodies that bind to antigenic epitopes common among multiple *H. influenzae* subtypes but unique with respect to any other antigenic epitopes. The antibodies may be polyclonal antibodies, monoclonal antibodies, antibody fragments which retain their ability to bind their unique epitope (*e.g.*, Fv, Fab and F(ab)2 fragments), single chain antibodies and human or humanized antibodies. Antibodies may be generated by techniques standard in the art.

In vitro complement mediated bactericidal assay systems (Musher *et al.*, *Infect. Immun.* 39: 297-304, 1983; Anderson *et al.*, *J. Clin. Invest.* 51: 31-38, 1972) may be used to measure the bactericidal activity of antibodies that specifically bind to NTHi SapA, SapB, SapC, SapD, SapF, SapZ and NTHi 110 kDa OMP polypeptides. Further data on the ability of NTHi 110 kDa OMP protein and peptides thereof elicit a protective antibody response may be generated by using animal models of infection such as the chinchilla model system described herein.

The present invention provides for antibodies specific for the NTHi polypeptides of the present invention and fragments thereof, which exhibit the ability to kill both *H. influenzae* bacteria and to protect humans from NTHi infection. The present invention also provides for antibodies specific for the NTHi polypeptides of the invention that reduce the virulence, inhibit adherence, inhibit cell division, and/or inhibit penetration of *H. influenzae* bacteria into the epithelium or enhance phagocytosis of the *H. influenzae* bacteria.

It is also possible to confer short-term protection to a host by passive immunotherapy by the administration of pre-formed antibody against an epitope or epitopes of the NTHi SapA, SapB, SapC, SapD, SapF, SapZ proteins and NTHi 110 kDa OMP protein. Thus, the contemplated vaccine formulations can be used to produce antibodies for use in passive immunotherapy. Human immunoglobulin is preferred in human medicine because a heterologous immunoglobulin may provoke an immune response to its foreign immunogenic components. Such passive immunization could be used on an emergency basis for immediate protection of unimmunized individuals exposed to special risks. Alternatively, these antibodies can be used in the production of anti-idiotypic antibody, which in turn can be used as an

antigen to stimulate an immune response against one or more of the NTHi SapA, SapB, SapC, SapD, SapF, SapZ proteins and NTHi 110 kDa OMP protein.

The invention contemplates methods of eliciting an immune response to NTHi in an individual. These methods include immune responses that kill the NTHi bacteria and immune responses which block *H. influenzae* attachment to cells or *H. influenzae* proliferation. In one embodiment, the methods comprise a step of administering an immunogenic dose of a composition comprising one or more of the NTHi SapA, SapB, SapC, SapD, SapF, SapZ and NTHi 110 kDa OMP proteins or peptides thereof. In another embodiment, the methods comprise administering an immunogenic dose of a composition comprising a cell expressing one or more of the NTHi SapA, SapB, SapC, SapD, SapF, SapZ and NTHi 110 kDa OMP proteins or peptides thereof. In yet another embodiment, the methods comprise administering an immunogenic dose of a composition comprising a polynucleotide encoding one or more of the NTHi SapA, SapB, SapC. SapD, SapF, SapZ and NTHi 110 kDa OMP proteins or peptides thereof. The polynucleotide may be a naked polynucleotide not associated with any other nucleic acid or may be in a vector such as a plasmid or viral vector (*e.g.*, adeno-associated virus vector or adenovirus vector). Administration of the compositions may be by routes standard in the art, for example, parenteral, intravenous, oral, buccal, nasal, pulmonary, rectal, or vaginal. The methods may be used in combination in a single individual. The methods may be used prior or subsequent to NTHi infection of an individual.

An "immunogenic dose" of a composition of the invention is one that generates, after administration, a detectable humoral and/or cellular immune response in comparison to the immune response detectable before administration or in comparison to a standard immune response before administration. The invention contemplates that the immune response resulting from the methods may be protective and/or therapeutic. For example , an "immunogenic dose" is a dose that is adequate to produce antibody and/or T cell immune responses to NTHi. In some embodiments the immune response protects said individual from NTHi infection, particularly NTHi infection of the middle ear, nasopharynx and/or lower airway. Also provided are methods whereby such immune response slows bacterial replication. The immune response may be induced therapeutically or prophylactically and may take the form of antibody immunity or cellular immunity such as that arising from CTL or CD4+ T

cells. The NTHi protein or an antigenic peptide thereof may be fused with co-protein which may not by itself induce antibodies, but is capable of stabilizing the first protein and producing a fused protein which will have immunogenic and protective properties. Thus fused recombinant protein, may further comprise an antigenic co-protein, such as glutathione-S-transferase (GST) or beta-galactosidase, relatively large co-proteins that solubilize the protein and facilitate production and purification thereof. Moreover, the co-protein may act as an adjuvant in the sense of providing generalized stimulation of the immune system. The co-protein may be attached to either the amino or carboxy terminus of the first protein.

The invention correspondingly provides compositions suitable for eliciting an immune response to NTHi infection, wherein antibodies elicited block binding of NTHi bacterium to the host's cells, reduce the virulence, inhibit adherence, inhibit cell division, and/or inhibit penetration of *H. influenzae* bacteria into the epithelium or enhance phagocytosis of the *H. influenzae* bacteria. The compositions comprise one or more NTHi SapA, SapB, SapC, SapD, SapF, SapZ and NTHi 110 kDa OMP proteins or peptides thereof, cells expressing one or more NTHi SapA, SapB, SapC. SapD, SapF, SapZ and NTHi 110 kDa OMP proteins, or polynucleotides encoding one or more NTHi SapA, SapB, SapC, SapD, SapF, SapZ proteins and NTHi 110 kDa OMP protein. The compositions may also comprise other ingredients such as carriers and adjuvants.

The invention includes methods of blocking binding of NTHi bacteria to host cells in an individual. The methods comprise inducing and/or administering antibodies of the invention that block binding of NTHi cellular attachment, reduce the virulence, inhibit adherence, inhibit cell division, and/or inhibit penetration of *H. influenzae* bacteria into the epithelium or enhance phagocytosis of the *H. influenzae* bacteria. Alternatively, administration of one or more small molecules that block binding of NTHi cell attachment is contemplated. *In vitro* assays may be used to demonstrate the ability of an antibody, polypeptide or small molecule of the invention to block NTHi cell attachment.

Pharmaceutical compositions comprising antibodies of the invention, or small molecules of the invention that block NTHi cellular attachment, reduce the virulence, inhibit adherence, inhibit cell division, and/or inhibit penetration of *H. influenzae* bacteria into the epithelium or enhance phagocytosis of the *H. influenzae*

bacteria are provided. The pharmaceutical compositions may consist of one of the foregoing active ingredients alone, may comprise combinations of the foregoing active ingredients or may comprise additional active ingredients used to treat bacterial infections. The pharmaceutical compositions may comprise one or more additional ingredients such as pharmaceutically effective carriers. Dosage and frequency of the administration of the pharmaceutical compositions are determined by standard techniques and depend, for example, on the weight and age of the individual, the route of administration, and the severity of symptoms. Administration of the pharmaceutical compositions may be by routes standard in the art, for example, parenteral, intravenous, oral, buccal, nasal, pulmonary, rectal, or vaginal.

Also provided by the invention are methods for detecting NTHi infection in an individual. In one embodiment, the methods comprise detecting one or more NTHi SapA, SapB, SapC, SapD, SapF, SapZ and NTHi 110 kDa OMP proteins in a sample using primers or probes that specifically bind to the polynucleotides. Detection of the polynucleotides may be accomplished by numerous techniques routine in the art involving, for example, hybridization and PCR.

The antibodies of the present invention may also be used to provide reagents for use in diagnostic assays for the detection of one or more NTHi SapA, SapB, SapC, SapD, SapF, SapZ and NTHi 110 kDa OMP proteins or peptides thereof in various body fluids of individuals suspected of *H. influenzae* infection. In another embodiment, the NTHi SapA, SapB, SapC, SapD, SapF, SapZ or NTHi 110 kDa OMP protein or peptides thereof of the present invention may be used as antigens in immunoassays for the detection of NTHi in various patient tissues and body fluids including, but not limited to: blood, serum, ear fluid, spinal fluid, sputum, urine, lymphatic fluid and cerebrospinal fluid. The antigens of the present invention may be used in any immunoassay system known in the art including, but not limited to: radioimmunoassays, ELISA assays, sandwich assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, fluorescent immunoassays, protein A immunoassays and immunoelectrophoresis assays.

Vaccines and Chemotherapeutic Targets

As noted above, an aspect of the invention relates to a method for inducing an immune response in an individual, particularly a mammal, that comprises

inoculating the individual with one or more NTHi SapA, SapB, SapC, SapD, SapF, SapZ and NTHi 110 kDa OMP proteins or an antigenic peptides thereof. The present invention also provides for vaccine formulations that comprise one or more immunogenic recombinant NTHi SapA, SapB, SapC, SapD, SapF, SapZ and NTHi 110 kDa OMP proteins or peptides thereof together with a suitable carrier. The NTHi SapA, SapB, SapC, SapD, SapF, SapZ or NTHi 110 kDa OMP protein or peptides thereof are contemplated as vaccine candidates and/or targets of chemotherapy.

Since the protein may be broken down in the stomach, it is preferably administered parenterally, including, for example, administration that is subcutaneous, intramuscular, intravenous, or intradermal. Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the bodily fluid, preferably the blood, of the individual; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

A. Peptide Vaccines

Peptide therapeutic agents, such as peptide vaccines, are well known in the art and are of increasing use in the pharmaceutical arts. Consistent drawbacks to the parenteral administration of such peptide compounds have been the rapidity of breakdown or denaturation. Infusion pumps, as well as wax or oil implants, have been employed for chronic administration of therapeutic agents in an effort to both prolong the presence of peptide-like therapeutic agents and preserve the integrity of such agents. Furthermore, the peptide-like agent should (with particular reference to each epitope of the peptide-like agent) ideally maintain native state configuration for an extended period of time and additionally be presented in a fashion suitable for triggering an immunogenic response in the challenged animal.

The NTHi polypeptides or peptides thereof of the invention can be prepared in a number of conventional ways. The short peptides sequences can be prepared by chemical synthesis using standard means. Particularly convenient are solid phase techniques (see, *e.g.*, Erikson *et al.*, *The Proteins* (1976) v. 2, Academic Press, New York, p. 255). Automated solid phase synthesizers are commerically available. In addition, modifications in the sequence are easily made by substitution, addition or omission of appropriate residues. For example, a cysteine residue may be added at the carboxy terminus to provide a sulfhydryl group for convenient linkage to a carrier protein, or spacer elements, such as an additional glycine residue, may be incorporated into the sequence between the linking amino acid at the C-terminus and the remainder of the peptide. The short NTHi peptides can also be produced by recombinant techniques. The coding sequence for peptides of this length can easily be synthesized by chemical techniques, *e.g.*, the phosphotriester method described in Matteucci *et al.*, *J Am Chem Soc.*, 103: 3185 (1981).

Where some of the NTHi peptide sequences contemplated herein may be considered too small to be immunogenic, they may be linked to carrier substances in order to confer this property upon them. Any method of creating such linkages known in the art may be used. Linkages can be formed with heterobifunctional agents that generate a disulfide link at one functional group end and a peptide link at the other, such as a disulfide amide forming agent, *e.g.*, N-succidimidyl-3-(2-pyridyldithio) proprionate (SPDP) (See, *e.g.*, Jansen *et al.*, *Immun. Rev.* 62:185, 1982) and bifunctional coupling agents that form a thioether rather than a disulfide linkage such as reactive esters of 6-maleimidocaproic acid, 2-bromoacetic acid, 2-iodoacetic acid, 4-(N-maleimido-methyl) cyclohexane-1-carboxylic acid and the like, and coupling agents which activate carboxyl groups by combining them with succinimide or 1-hydroxy-2-nitro-4-sulfonic acid, for sodium salt such as succinimmidyl 4-(N-maleimido-methyl) cyclohexane-1-carobxylate (SMCC).

B. Vaccine Compositions and Administration

A priming dose of an immunogenic composition of the invention may be followed by one or more booster exposures to the immunogen. (Kramp *et al.*, *Infect. Immun.*, 25: 771-773, 1979; Davis *et al.*, *Immunology Letters*, 14: 341-8 1986 1987). moreover, examples of proteins or polypeptides that could beneficially enhance the immune response if co-administered include cytokines (*e.g.*, IL-2, IL-12,

GM-CSF), cytokine-inducing molecules (*e.g.* Leaf) or costimulatory molecules. Helper (HTL) epitopes could be joined to intracellular targeting signals and expressed separately from the CTL epitopes. This would allow direction of the HTL epitopes to a cell compartment different than the CTL epitopes. If required, this could facilitate more efficient entry of HTL epitopes into the MHC class II pathway, thereby improving CTL induction. In contrast to CTL induction, specifically decreasing the immune response by co-expression of immunosuppressive molecules (*e.g.* TGF-β) may be beneficial in certain diseases. .

Ideally, an immunogen will exhibit two properties; the capacity to stimulate the formation of the corresponding antibodies and the propensity to react specifically with these antibodies. Immunogens bear one or more epitopes that are the smallest part of an immunogen recognizable by the combing site of an antibody. In particular instances, immunogen, fractions of immunogens or conditions under which the immunogen is presented are inadequate to precipitate the desired immune response resulting in insufficient immunity. This is often the case with peptides or other small molecules used as immunogens. Other substances such as immunomodulators (*e.g.*, cytokines such as the interleukins) may be combined in vaccines as well.

The vaccine art recognizes the use of certain substances called adjuvants to potentate an immune response when used in conjunction with an immunogen. Adjuvants are further used to elicit an immune response that is faster or greater than would be elicited without the use of the adjuvant. In addition, adjuvants may be used to create an immune response using less immunogen than would be needed without the inclusion of adjuvant, to increase production of certain antibody subclasses that afford immunogenic protection or to enhance components of the immune response (*e.g.*, humoral, cellular). Known adjuvants include emulsions such as Freund's Adjuvants and other oil emulsions, *Bordetella pertussis*, MF59, purified saponin from *Quillaja saponin* (QS21), aluminum salts such as hydroxide, phosphate and alum, calcium phosphate, (and other metal salts), gels such as aluminum hydroxide salts, mycobacterial products including muramyl dipeptides, solid materials, particles such as liposomes and virosomes. Examples of natural and bacterial products known to be used as adjuvants include monophosphoryl lipid A (MPL), RC-529 (synthetic MPL-like acylated monosaccharide), OM-174 which is a

lipid A derivative from *E. coli.*, holotoxins such as cholera toxin (CT) or one of its derivatives, pertussis toxin (PT) and heat-labile toxin (LT) of *E. coli* or one of its derivatives, and CpG oligonucleotides. Adjuvant activity can be affected by a number of factors, such as carrier effect, depot formation, altered lymphocyte recirculation, stimulation of T-lymphocytes, direct stimulation of B-lymphocytes and stimulation of macrophages.

Vaccines are typically prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, *e.g.*, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants that enhance the effectiveness of the vaccine. The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25-70%.

Vaccines may also be administered through transdermal routes utilizing jet injectors, microneedles, electroporation, sonoporation, microencapsulation, polymers or liposomes, transmucosal routes and intranasal routes using nebulizers, aerosols and nasal sprays. Microencapsulation using natural or synthetic polymers such as starch, alginate and chitosan, D-poly L-lactate (PLA), D-poly DL-lactic-coglycolic microspheres, polycaprolactones, polyorthoesters, polyanhydrides and polyphosphazanes are useful for both transdermal and

transmucosal administration. Polymeric complexes comprising synthetic poly-ornithate, poly-lysine and poly-arginine or amiphipathic peptides are useful for transdermal delivery systems. In addition, due to their amphipathic nature, liposomes are contemplated for transdermal, transmucosal and intranasal vaccine delivery systems. Common lipids used for vaccine delivery include N-(1)2,3-dioleyl-dihydroxypropyl)-*N,N,N*, - trimethylammonium-methyl sulfate (DOTAP), dioleyloxy-propyl- trimethylammonium chloride (DOTMA), dimystyloxypropyl-3-dimethyl-hydroxyethyl ammonium (DMRIE), dimethyldioctadecyl ammonium bromide (DDAB) and 9*N*(*N'*,*N*-dimethylaminoethane) carbamoyl) cholesterol (DC-Chol). The combination of helper lipids and liposomes will enhance up-take of the liposomes through the skin. These helper lipids include, dioeolphosphatidylethanolamine (DOPE), dilauroylphosphatidylethanolamine (DLPE), dimystristoylphosphatidylethanolamine (DMPE), dipalmitoyl phosphatidylethanolamine (DPPE). In addition, triterpenoid glycosides or saponins derived from the Chilean soap tree bark (*Quillaja saponaria*) and chitosan (deacetylated chitan) have been contemplated as useful adjuvants for intranasal and transmucosal vaccine delivery.

The proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, *e.g.*, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, *e.g.*, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, and procaine.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per individual. Suitable regimes for initial administration and

booster shots are also variable, but are typified by an initial administration followed in one or three month intervals by a subsequent injection or other administration.

Upon immunization with a vaccine composition as described herein, the immune system of the host responds to the vaccine by producing large amounts of CTLs specific for the desired antigen, and the host becomes at least partially immune to later infection, or resistant to developing chronic infection. Vaccine compositions containing one or more NTHi SapA, SapB, SapC, SapD, SapF, SapZ and NTHi 110 kDa OMP proteins or peptides thereof are administered to a patient susceptible to or otherwise at risk of bacterial infection or cancer to elicit an immune response against the antigen and thus enhance the patient's own immune response capabilities. Such an amount is defined to be an "immunogenically effective dose. " In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally range from about 1.0 μg to about 5000 per 70-kilogram patient, more commonly from about 10 to about 500 mg per 70 kg of body weight. For therapeutic or immunization purposes, the NTHi SapA, SapB, SapC, SapD, SapF, SapZ or NTHi 110 kDa OMP protein or peptides thereof may also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into an acutely or chronically infected host or into a noninfected host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host CTL response.

Humoral immune response may be measured by many well-known methods, such as Single Radial Immunodiffussion Assay (SRID), Enzyme Immunoassay (EIA) and Hemagglutination Inhibition Assay (HAI). In particular, SRID utilizes a layer of a gel, such as agarose, containing the immunogen being tested. A well is cut in the gel and the serum being tested is placed in the well. Diffusion of the antibody out into the gel leads to the formation of a precipitation ring whose area is proportional to the concentration of the antibody in the serum being tested. EIA, also known as ELISA (Enzyme Linked Immunoassay), is used to determine total antibodies in the sample. The immunogen is adsorbed to the surface of a microtiter plate. The test serum is exposed to the plate followed by an enzyme linked immunoglobulin, such as IgG. The enzyme activity adherent to the plate is

quantified by any convenient means such as spectrophotometry and is proportional to the concentration of antibody directed against the immunogen present in the test sample. HAI utilizes the capability of an immunogen such as viral proteins to agglutinate chicken red blood cells (or the like). The assay detects neutralizing antibodies, *i.e.*, those antibodies able to inhibit hemagglutination. Dilutions of the test sera are incubated with a standard concentration of immunogen, followed by the addition of the red blood cells. The presence of neutralizing antibodies will inhibit the agglutination of the red blood cells by the immunogen. Tests to measure cellular immune response include determination of delayed-type hypersensitivity or measuring the proliferative response of lymphocytes to target immunogen.

Assays for measuring T-cell response are well known in the art. For example, T-cell response can be measured using delayed-type hypersensitivity testing, flow cytometry using peptide major histocompatibility complex tetramers, lymphoproliferation assay, enzyme-linked immunosorbant assay, enzyme-linked immunospot assay, cytokine flow cytometry, direct cytotoxicity assay, measurement of cytokine mRNA by quantitative reverse transcriptase polymerase chain reaction, and limiting dilution analysis. (See Lyerly , *Semin Oncol.*, 30(3 Suppl 8):9-16, 2003).

Nontypeable *Haemophilus influenzae* (NTHi)

H. influenzae is a small, nonmotile gram negative bacterium. Unlike other *H. influenzae* strains, the nontypable *H. influenzae* (NTHi) strains lack a polysaccharide capsule and are sometimes denoted as "nonencapsulated." NTHi strains are genetically distinct from encapsulated strains and are more heterogenous than the type b *H. influenzae* isolates. NTHi presents a complex array of antigens to the human host. Possible antigens that may elicit protection include OMPs, liposaccharises, lipoproteins, adhesion proteins and noncapsular proteins.

Humans are the only host for *H. influenzae*. NTHi strains commonly reside in the middle ear, upper respiratory tract including the nasopharynx and the posterior oropharynx, the lower respiratory tract and the female genital tract. NTHi causes a broad spectrum of diseases in humans, including but not limited to, otitis media, pneumonia, sinusitis, septicemia, endocarditis, epiglottitis, septic arthritis, meningitis, postpartum and neonatal infections, postpartum and neonatal sepsis, acute and chromic salpingitis, epiglottis, pericardis, cellulitis, osteomyelitis, endocarditis, cholecystitis, intraabdominal infections, urinary tract infection, mastoiditis, aortic

graft infection, conjunctitivitis, Brazilian purpuric fever, occult bacteremia and exacerbation of underlying lung diseases such as chronic bronchitis, bronchietasis and cystic fibrosis.

Epidemiologic studies of NTHi have indicated that the strains are heterogeneous with respect to outer membrane protein profiles (Barenkamp *et al., Infect. Immun.,* 36: 535-40, 1982), enzyme allotypes (Musser *et al., Infect. Immun.,* 52: 183-191, 1986), and other commonly used epidemiologic tools. There have been several attempts to subtype NTHi, but none of the methodologies have been totally satisfactory. The outer-membrane protein composition of NTHi consists of approximately 20 proteins. All NTHi strains contain two common OMP's with molecular weights of 30,000 and 16,600 daltons. NTHi strains may be subtyped based on two OMP's within the 32,000-42,000 dalton range. The NTHi lipopolysaccharide profile is fundamentally different than the enteric Gram-negative bacteria and separates into several distinct bands less than 20,000 daltons in size.

A prototype NTHi isolate is the low passage isolate 86-028NP which was recovered from a child with chronic otitis media. This strain has been well characterized *in vitro* (Bakaletz *et al., Infect. Immun.,* 53: 331-5, 1988; Holmes *et al., Microb. Pathog.,* 23: 157-66, 1997) as well as in the chinchilla OM model (described herein) (Bakaletz *et al., Vaccine,* 15: 955-61, 1997; Suzuki *et al., Infect. Immun.,* 62: 1710-8, 1994; DeMaria *et al., Infect. Immun.,* 64: 5187-92, 1996). The 86-028NP strain was used, as described herein, to identify genes that are up-regulated in expression in the chinchilla model of otitis media and genes that are necessary for NTHi survival in the chinchilla middle ear.

The NTHi strain 86-026NP was deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110, on October 16, 2001 and assigned accession no. PTA-4764.

Signature-Tag Mutagenesis Strategy

The signature tag mutagenesis strategy (STM) has been employed to identify genes that are required for bacterial survival during infection in a number of systems. In this strategy, a series of mutants are constructed by random transposon mutagenesis. Each mutant was uniquely tagged with an oligonucleotide sequence that enables tag-specific identification of genes that alter virulence properties of a

microorganism when mutated. The pool of mutants is then used to infect the experimental animal ('input pool'). After an appropriate period of time has elapsed, the surviving infecting organisms are recovered ('recovery pool').

Herein, the Tn*903* kanamycin resistance gene was cloned into the *Eco*RI site of the pUC-based mini-Tn5 construction vector EZ::TN pMOD-2 (Epicentre). Oligonucleotide tags were prepared using the strategy of Nelson *et al.* (*Genetics*, 157: 935-47, 2001) and cloned into the *Kpn*I site of the modified EZ::TN pMOD-2 vector. Individual tags were characterized to confirm that they hybridized uniquely. Seventy-eight unique tags were saved. Chromosomal DNA from strain 86-028NP was mutagenized with 38 individual tag-containing mini-Tn*5* elements *in vitro*, gaps repaired with T4 polymerase and ligase, then mutagenized DNA was transformed back into strain 86-028NP using the M-IV method. Mutants were selected for growth on kanamycin-containing media. A signature tagged library containing 2500 clones was screened for mutants defective in their ability to survive in the chinchilla middle ear. The genes disrupted by the mini-Tn5 elements in avirulent mutants were identified by sequencing DNA flanking the mini-Tn5 elements. Template was prepared using single primer PCR strategy.

This analysis identified an avirulent clone containing a mutation in *sapF*. The *sap* operon has been shown in other systems to confer resistance to cationic antimicrobial peptides (Lopez-Solanilla *et al., Plant Cell,* 10(6): 917-24, 1998; McCoy *et al., Antimicrobiol. Agent Chemother.*, 45(7): 2030-7, 2001: Parra-Lopez *et al., EMBO J.,* 12(11): 4053-62, 1993). *In vitro*, the *H. influenzae sapF* mutant is more sensitive to cationic peptides suggesting that resistance to cationic peptides involved in innate immunity may be an important virulence determinant for *H. influenzae* in otitis media.

DFI Strategy

A differential fluorescence induction (DFI) strategy may be used to identify NTHi genes induced during OM in a chinchilla animal model. Several methods have been developed to identify bacterial genes that contribute to the virulence of an organism during infection. Such methods include *in vivo* expression technology (IVET) in which bacterial promoters regulate the expression of gene(s) required for synthesis of essential nutrients required for survival in the host; DNA microarray technology to globally screen for transcriptionally active genes, and DFI

which uses FACS analysis to select for transcriptionally active promoters (Chiang *et al., Annu. Rev. Microbiol.*, 53: 129-154, 1999). DFI is a high-throughput method that allows for the identification of differentially regulated genes regardless of the basal level of expression and does not exclude those that are essential for growth *in vitro*.

DFI has been successfully utilized in many microorganisms. For example, a GFP reporter system and flow cytometry was used to study mycobacterial gene expression upon interaction with macrophages (Dhandayuthapani *et al., Mol. Microbiol.*, 17: 901-912, 1995). A promoter trap system was used to identify genes whose transcription was increased when *Salmonellae* were subjected to environments simulating *in vivo* growth and when internalized by cultured macrophage-like cells (Valdivia and Falkow, *Mol. Microbiol.*, 22: 367-378, 1996; Valdivia and Falkow, *Science,* 277: 2007-2011, 1997; Valdivia and Falkow, *Curr. Opin. Microbiol.*, 1: 359-363, 1998). In addition, DFI has been used to identify promoters expressed in *S. pneumoniae* and *S. aureus* when grown under varied *in vitro* conditions simulating infection (Marra *et al., Infect. Immun.,* 148: 1483-1491, 2002; Schneider *et al., Proc. Natl. Acad. Sci. U.S.A.,* 97: 1671-1676, 2000). In addition, DFI has been utilized to study gene regulation in *Bacillus cereus* in response to environmental stimuli (Dunn and Handelsman, *Gene*, 226: 297-305, 1999), in *S. pneumoniae* in response to a competence stimulatory peptide (Bartilson *et al., Mol. Microbiol.*, 39: 126-135, 2001), and upon interaction with and invasion of host cells in *Bartonella henselae* (Lee and Falkow, *Infect. Immun.*, 66: 3964-3967, 1998), *Listeria monocytogenes* (Wilson *et al., Infect. Immun.*, 69: 5016-5024, 2001), *Brucella abortus* (Eskra *et al., Infect. Immun.*, 69: 7736-7742, 2001), and *Escherichia coli* (Badger *et al., Mol. Microbiol.*, 36: 174-182, 2000).

Animal Model

The chinchilla model is a widely accepted experimental model for OM. In particular, a chinchilla model of NTHi-induced OM has been well characterized (Bakaletz *et al., J. Infect. Dis.,* 168: 865-872, 1993; Bakaletz and Holmes, *Clin. Diagn. Lab. Immunol.*,4: 223-225, 1997; Suzuki and Bakaletz, *Infect. Immun.*, 62: 1710-1718, 1994), and has been used to determine the protective efficacy of several NTHi outer membrane proteins, combinations of outer membrane proteins, chimeric synthetic peptide vaccine components, and adjuvant formulations as vaccinogens

against OM (Bakaletz *et al., Vaccine*, 15: 955-961, 1997; Bakaletz *et al., Infect. Immun.*, 67: 2746-2762, 1999; Kennedy *et al., Infect. Immun.*, 68: 2756-2765, 2000).

In particular, there is a unique *in vivo* model wherein adenovirus predisposes chinchillas to *H. influenzae*-induced otitis media, which allowed for the establishment of relevant cell, tissue and organ culture systems for the biological assessment of NTHi (Bakaletz *et al., J. Infect. Dis.*, 168: 865-72, 1993; Suzuki *et al., Infect. Immunity* 62: 1710-8, 1994). Adenovirus infection alone has been used to assess for the transudation of induced serum antibodies into the tympanum (Bakaletz *et al., Clin. Diagnostic Lab Immunol.*, 4(2): 223-5, 1997) and has been used as a co-pathogen with NTHi, to determine the protective efficacy of several active and passive immunization regimens targeting various NTHi outer membrane proteins, combinations of OMPs, chimeric synthetic peptide vaccine components, and adjuvant formulations as vaccinogens against otitis media (Bakaletz *et al., Infect Immunity*, 67(6): 2746-62, 1999; Kennedy *et al., Infect Immun.*, 68(5): 2756-65, 2000; Novotny *et al., Infect Immunity* 68(4): 2119-28, 2000; Poolman *et al., Vaccine* 19 (Suppl. 1): S108-15, 2000).

Brief Description of Figures

Figure 1 depicts identification of attenuated A1 clone (circled) by comparative hybridization of signature tags present in the input pool (A) and the recovery pool (B).

Figure 2 depicts that interruption of the *sapF* gene by the miniTn5 transposon had no polar effect on the downstream *sapZ* gene in the *sapF*::mTn5 mutant. RT-PCR analysis showing transcription of the *sapZ* gene (A). Insertion of miniTn5 in the *sapF* gene near the 3' end (B). Short arrows are RT-PCR primers. Lines represent RT-PCR products.

Figure 3 depicts RT-PCR analysis showing cotranscription of the *sapABCDFZ* genes as a single polycistronic mRNA. Transcriptional profile of the NTHi *sap* genes when grown in the sBHI media (A), and the computer predicted NTHi *sap* operon (B). Short arrows are RT-PCR primers. Lines represent RT-PCR products.

Figure 4 depicts the gene order of the NTHi *sap* gene cluster. RT-PCR analysis demonstrates these genes are transcribed as an operon.

Figure 5 depicts the sensitivity of NTHi bacterium with the *sapA::kan* mutation or the parental NTHi strain to killing induced by recombinant chinchilla beta-defensin-1 (cBD-1).

Figure 6 depicts the relative bacterial counts in the chinchilla middle ear after inoculation of equal parts *sapA::kan* mutant NTHi and the parental NTHi strain. This plot depicts the inability of the *sapA::kan* mutant to survive in the middle ear while the parental strain maintained high bacterial counts.

Figure 7 depicts the ability of the *sapA::kan* mutant to survive when inoculated alone in the chinchilla middle ear (top panel) or in the chinchilla nasopharynx (bottom panel). These plots depict the inability of the *sapA::kan* mutant to survive *in vivo* while the parental strain maintained high bacterial counts.

Detailed Description

The following examples illustrate the invention wherein Example 1 describes construction of a signature-tagged mutagenesis library and identification of avirulent NTHi clones, Example 2 describes the characterization of the avirulent NTHi clone A1, Example 3 describes the *in vitro* phenotypic characterization of the NTHi *sapF*::mTn5 mutant, and Example 4 describes the OMP profile of the NTHi *sapF*::mTn5 mutant.

Example 1
Construction of the STM Library

An attenuated NTHi mutant was identified by signature-tagged mutagenesis (STM) using the transbullar chinchilla model of OM. The NTHi, strain 86-028NP, was mutagenized by miniTn5 transposons marked with unique signature tags to construct an STM library. A panel of signature-tagged miniTn5 transposons was constructed by cloning an EcoRI cassette containing the Tn903 kanamycin resistance gene into the EcoRI site and a signature tag sequence into the KpnI site within the transposon of the Epicentre EZ::TN pMOD<MCS> Transposon Construction Vector. To ensure that the signature tag sequences give a strong hybridization signal and do not cross hybridize to other tags, the signature tag sequences were screened by dot blot hybridization. To adapt the Epicentre miniTn5 *in vitro* transposition mutagenesis system to strain 86-028NP, single stranded gaps generated by the transposase in the chromosomal DNA were repaired using DNA

polymerase and ligase. The transposon inserted DNA was transformed back into the parent strain using M-IV transformation method described in Herriott *et al.* (*J. Bacterial.* 101: 513-6, 1970). The individual kanamycin resistant clones with unique tags were assembled into 96 well plates for animal screening. Southern blot analysis was performed to confirm random and single insertion of the transposon in the STM mutants.

A pool of 38 STM mutants containing unique signature tags were directly inoculated into the middle ear cavity of a chinchilla at a concentration of 1.0×10^6 cfu/ear. The chinchilla was monitored for OM development and formation of effusions in the middle ear over a period of 48 hours by otoscopy and tympanometry. Effusions were removed by epitympanic taps and plated on chocolate agar plates supplanted with kanamycin to recover the NTHi mutants that survived in the middle ear. Bacteria recovered after two days of inoculation were selected as the recovery pool, at which time point the proliferation of NTHi cells in the middle ear reached a peak level during the course of OM development.

Bacterial genomic DNA isolated from the input and recovery pool was used as template for PCR amplification of signature tags. The input and recovery probes were hybridized to membranes spotted with each signature tag PCR product or oligonucleotide in quadruplicate. By comparing the input and recovery hybridization patterns as depicted in Fig. 1, attenuated mutants containing signature tags were identified within the input pool but not in the recovery pool. The mutant carrying the A1 tag (circled in Fig. 1) was cleared from the middle ear in two other independent STM animal experiments confirming that this mutant was attenuated *in vivo*. This mutant was subjected for further characterization as described below.

Example 2
Characterization of the Attenuated A1 Clone

Sequence analysis was carried out on the transposon interrupted DNA locus in the attenuated strain using standard methods in the art. Southern blot analysis showed that a 6 kb EcoRV restricted genomic DNA fragment of the mutant of interest contained the transposon interrupted gene. The EcoRV restricted genomic DNA fragments were cloned into the pBluescript plasmid, and the transposon containing clone, designated pBlueA1, was isolated using marker rescue from LB agar plates supplemented with kanamycin. The 6 kb insert of the pBlueA1plasmid was sequenced

and the resulting DNA sequence data were searched against NCBI databases using the BLASTX and BLASTN algorithms. Contigs were assembled using SeqmanII software (DNASTAR Inc.). As shown in Fig. 2, sequence analysis indicated that the transposon was inserted 165 bp from the 3'-end of the *sapF* gene, thus this attenuated mutant was designated as "*sapF*::mTn5." The coding sequence of the kanamycin resistance gene is in the same orientation as the *sapF* gene.

A search of the *H. influenzae* Rd genome using the *sapF* DNA, identified the *Haemophilus sap* gene cluster containing 6 open reading frames (ORFs) in the order of *sapABCDFZ*, where the *sapF* was the fifth gene of the cluster followed by a hypothetical protein HI1643 which we designated "*sapZ*" in this study. This study, utilized the genomic sequencing NTHi strain 86-028NP and a three-fold coverage contig assembly. Part of the *sap* operon was present in the contigs (Contigs 512 and 324; SEQ ID NO: 16-17). A pair of primers were designed according to the contig sequences to PCR amplify the whole *sap* operon from strain 86-028NP. Sequence comparison analysis showed that the *sap* operon of strain 86-028NP had 98% identity as that of strain Rd, and the *sap* genes were organized in the same way. The polynucleotide sequence of the *sap* operon genes (*sapA, sapB, sapC, sapD, sapF,* and *sapZ*) are set out as SEQ ID NOS: 1-6, respectively. The amino acid sequences of the *sap* operon gene products, SapA, SapB, SapC, SapD, SapF and SapZ, are set out as SEQ ID NOS: 7-12 respectively.

The *sapF* gene contains an ATP-binding domain and may share translocation ATPase activity with the *sapD* gene, shown to be up-regulated in response to iron and may play a role in potassium uptake via the TRK system (Harms *et al., Microbiology* 147: 2991-3003, 2001; Paustian *et al. J. Bacteriol*, 184:6714-20, 2002) The *sapZ* gene is unique to *Haemophilus*. SapZ is predicted to be a transmembrane protein with gene homologs in *sap* operon-containing bacteria, *P. multocida, S. typhimurium, S. enterica*, and *E. coli 0157:H7*, and in *Neisseria meningitidis* and *Pseudomonas aeruginosa*, which do not contain a *sap* operon. In bacteria containing the described *sap* system, however, *sapZ* is not located near the *sap* operon in the bacterial genome. The NTHi *sap* operon locus is organized as a single operon containing 6 genes as displayed in Fig. 4 and this gene locus was upregulated *in vivo* as determined by quantitative RT-PCR.

DNA sequence analysis indicated that the coding sequences of the 86-028NP 6 *sap* genes were located on the same DNA strand with very few non-coding bases between the ORFs (Fig. 3). When the *sap* gene cluster was scanned for transcriptional terminators (GCG Wisconsin package v. 10), one typical rho-independent terminator as a stem-loop structure followed by polyU sequence was found downstream of the *sapZ* gene. Therefore, the 6 NTHi *sap* genes were predicted to be organized in an operon structure and presumed to be co-transcribed as one polycistronic mRNA. The *sapZ* gene begins 11 nucleotides downstream of the end of the *sapF* gene and therefore it is highly likely that is co-transcribed with the *sap* gene cluster. To confirm this organization, RT-PCR was used to determine whether the region between the *sap* genes was transcribed. Each RT-PCR reaction utilized a primer from the 3' end of one gene and a primer from the 5' end of the following gene. If there was a PCR product, the two adjacent genes were cotranscribed. As amplicons were obtained from each junction region, all 6 *sap* genes were co-transcribed as one polycistronic mRNA (Fig. 3, upper panel), which was in agreement with the transcriptional property of the *sap* operon in *S. typhimurium* (Parra-Lopez *et al*, *supra*).

In order to determine whether insertion of the transposon prevented transcription of the downstream *sapZ* gene in the *sapF*::mTn5 mutant, a similar RT-PCR strategy using primers which annealed to the 3'-end of the *sapF* gene or the miniTn5 transposon and a primer which annealed to the 5'-end of the *sapZ* gene was employed. As depicted in Fig. 2, both primer sets gave positive results using *sapF*::mTn5 RNA as template demonstrating that there was detectable *sapZ* mRNA produced in the *sapF*::mTn5 mutant. The *sapZ* transcript in the mutant is presumably due to the absence of a transcriptional terminator downstream of the kanamycin resistance gene in the miniTn5 transposon. Thus, the attenuated phenotype of strain *sapF*::mTn5 was likely due to the *sapF* mutation but not the result of polar effect on the downstream *sapZ* gene.

Example 3

In vitro Phenotypic Characterization of the *sapF*::mTn5 Mutant

To ensure no secondary mutation in the original *sapF*::mTn5 mutant contributed to the various phenotypes of this mutant, the parent strain 86-028NP was transformed with the 6 kb EcoRV fragment containing the *sapF*::mTn5 allele from the

pBlueA1 plasmid.. The wild type *sapF* gene was replaced in this strain by homologous recombination with the *sapF*::mTn5 allele. One Km resistant clone was confirmed to harbor a miniTn5 interrupted *sapF* gene by PCR and Southern blot analysis. This clone was further characterized together with the *sapF*::mTn5 strain and designated Rc*sapF*::mTn5.

Since the *sap* mutants of *S. typhimurium* and *E. chrysanthemi* were reported to be hypersensitive to certain antimicrobial peptides, sensitivity to several commercial available cationic peptides against the NTHi parent and the *sapF* mutant strains was analyzed. Protamine displayed differential killing effect on the *sapF* mutants comparing to the parent strain. Broth minimal inhibitory concentration (MIC) analyses for protamine determined that the MIC of protamine for the *sapF*::mTn5 mutants was lower than that for the parent strain (0.2 mg/ml versus 0.4 mg/ml). Growth curve measurement under the same growth condition (aerobic growth in sBHI broth) demonstrated that the growth curves of the two mutant strains and the parent strain were identical. This analysis suggests that the two mutant strains do not possess a growth defect. Thus, the *sapF* gene product is not required for growth in enriched media, and the lack of growth of the *sapF* mutants at the lower protamine concentrations in sBHI broth was not due to a growth defect. Therefore, the *sapF* mutation may be responsible for the phenotype of increased sensitivity to protamine, and the *in vivo* attenuation property of the *sapF* mutant.

Example 4
OMP Profile for the *sapF*::mTn5 Mutant NTHi Strain

The *sapF* mutant displayed a minor variation of OMP profile in comparison with the parent strain. Sarkosyl insoluble OMPs of the three strains were prepared using differential detergent extraction as described in Filip et al., (*J. Bacteriol.* 115: 717-722, 1973), and separated in a 10% SDS-PAGE. Absence of a 110 kDa OMP band was consistently observed from several OMP preparations in both mutant strains compared to the parent strain. Both the original and reconstructed mutant exhibited this minor change of the OMP profile, suggesting that the loss of the high molecular protein in the outer membrane was not due to a secondary mutation in the original *sapF*::mTn5 mutant.

To determine the amino acid sequence of the 110 kDa OMP protein, a tryptic digest was performed. The 110 kDa protein was digested overnight at 37°C.

Subsequently the peptides (SEQ ID NOS: 22-39) were extracted, desalted (10%) using C18ziptip (Millipore), and analyzed by Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The peptide information is set out below in Table 1. The MALDI monoisotopic peaks were then searched in the NCBInr database using the Profound computer program.

Table 1

Peptide sequence	SEQ ID NO:	Residues	Computed mass	Meaured mass
FYAPGR	22	998-1003	709.354	709.342
LWQER	23	530-534	730.376	730.372
FGQSGFAIR	24	122-130	981.503	981.492
AGVYNLTNR	25	959-967	1006.519	1006.512
YITWDSAR	26	969-976	1010.482	1010.482
KYITWDSAR	27	968-976	1138.577	1138.582
EFARINNGTR	28	504-513	1176.599	1176.552
YDNIHYQPK	29	659-667	1176.556	1176.552
LSFNPTENHR	30	292-301	1213.583	1213.602
SRGQDLSYTLK	31	313-323	1266.656	1266.692
YETGVTVVEAGR	32	110-121	1279.640	1279.682
NPEDTYDIYAK	33	914-924	1327.593	1327.632
FTLAADLYEHR	34	302-312	1334.661	1334.722
ELFEGYGNFNNTR	35	157-169	1559.700	1559.802
TMVYGLGYDHPSQK	36	887-900	1594.744	1594.892
VEHNLQYGSSYNTTMK	37	556-571	1870.851	1870.972
GYATENNQSFNTLTLAGR	38	223-240	1955.933	1956.082
KGYATENNQSFNTLTLAGR	39	222-240	2084.028	2084.172

This analysis identified the 110 kDa OMP protein as *H. influenzae* hemoglobin binding protein (HGBA_HAEIN; Genebank Accession No. Q9KIV2 or closely related homologue) by the Emory Microchemical Facility. The amino acid sequence from HGBA_HAEIN (Q9KIV2) (SEQ ID NO: 15) was employed to query the 86-028NP genomic contig set using the TBLASTN algorithm. The translation of the compliment of nucleotides 2623 to 5358 of contig 516 (SEQ ID NO: 18) was a translated sequence that is closely related to amino acids 94 to 1013 of HGBA_HAEIN (SEQ ID NO: 15). Similarly, contig 411 (SEQ ID NO: 19) contains nucleic acid sequences whose translation is highly related to amino acids 59 to 148 of HGBA_HAEIN and less closely related to amino acids 147-969 of HGBA_HAEIN. Contig 2 (SEQ ID NO: 39) contains nucleic acid sequences whose translation is highly related to amino acids 1 to 122 of HGBA_HAEIN (SEQ ID NO: 15). Contigs

469 and 497 (SEQ ID NOS: 20 and 21) also contain sequences with homology to HGBA_HAEIN. The sequence similarity is summarized in Table 2 below. Additional sequence analysis identified the full length sequence of the NTHi 110 kDa OMP set out as SEQ ID NO: 41 that is encoded by the nucleic acid set out in SEQ ID NO: 40.

Table 2

NTHi Contig #	Translation of Nucleotides of Contig with identity	Identity to Amino acids of SEQ ID NO: 15	Total of number of amino acids with identity	Percent Identity
516	complement of 2623-5358	94-1013	752/928	81%
469	complement of 427-3462	59-1013	464/1043	44%
411	651-3263	147-969	358/900	39%
411	388-657	59-148	82/90	91%
497	3377-4069	60-286	71/235	30%
2	79-396	1-122	61/122	50%

The *sapF* gene is 810 base pairs in length (SEQ ID NO: 5) and encodes a 269 amino acid protein (SEQ ID NO: 11) with a predicted mass of a 30 kDa soluble cytoplasmic protein with a an isoelectric point of 6.5. Therefore it is unlikely that the biosynthesis or secretion of this 110 kDa high molecular mass OMP is associated with the *sapF* gene product. Many OMPs of gram negative pathogens are important virulent factors playing roles in different pathogenesis aspects, such as host cells interaction, adhesion, iron acquisition, antigenic drift. The absence of the 110 kDa OMP may also contribute to the lost virulence of the *sapF*::Tn5 mutant.

Example 5
Generation of a Non-polar, In-frame Mutant of NTHI sap operon

A set of clones with putative promoter activity *in vivo* were identified by differential fluorescence induction, and upregulated *in vivo* expression was confirmed by quantitative RT-PCR analysis as described in Mason *et al.* (*Infection and Immunity* 71: 3454-3462, 2003). A clone that contained sequence upstream of the *sapA* gene was isolated. This clone demonstrated up-regulated GFP fluorescence *in vivo* indicating increased transcription of the *sap* operon. SapA was predicted to localize to the periplasm due to its signal sequence and its sequence identity to periplasmic solute binding proteins involved in peptide transport. (Parra-Lopez *et al.*,

EMBO J. 12: 4053-62, 1993) It was predicted that a mutation in the *sapA* gene would disrupt the function of the *sap* operon, thereby demonstrating the involvement of SapA in survival in a chinchilla model of otitis media.

A non-polar mutation in the *sapA* gene was generated by insertion of a promoterless kanamycin resistance cassette as described in Menard et al. (*J. Bacteriol.*, 175: 5899-906, 1993). The mutant construction was verified by Southern blot analysis and the resulting mutant is denoted herein as "*sapA::kan* mutant".

Example 6
Properties of the *sapA::kan* Mutant

Defensins are known as important elements of innate immunity against microbial infections. In particular, beta-defensins function to protect the host against microbial infections such as Gram-negative bacteria infections. Recombinant chinchilla beta-defensin-1 (cBD-1), an antimicrobial peptide with homology to human beta-defensin-3, was used to assess the sensitivity of the *sapA::kan* mutant to antimicrobial protection.

For microbicidal assays, NTHI strain 86-028NP or its isogenic *sapA::kan* mutant were cultured to mid-log phase in brain heart infusion (BHI) broth supplemented with 2 μg NAD/ml and 2 μg hemin/ml (sBHI) or on chocolate agar. Static cultures of NTHI, *S. pneumoniae* and *E. coli* were incubated in 5% CO_2 at 37°C. Various concentrations of recombinant cBD-1 (2.5, 5.0, 10.0 and 20 μg/ml) were incubated for 1 hour at 37°C in 5% CO_2 with 1 x 10^4 microorganisms in 100 μl of 10 mM sodium phosphate buffer containing either 1% sBHI. Bacteria were serially diluted and plated onto chocolate agar and the CFU of surviving microorganisms per ml was determined following overnight incubation at 37°C in 5% CO_2. Percent killing of the bacteria from a minimum of three replicate assays per strain are presented as mean percent survival (± SD) relative to concentration of (r)cBD-1 in Fig. 5. As shown in Fig. 5, the *sapA::kan* mutant strain had enhanced sensitivity to killing induced by recombinant chinchilla beta-defensin-1 as compared to the parental NTHi strain.

Survival of the *sapA::kan* mutant was also assessed *in vivo*. To conduct these studies, a small inoculum of either the parental NTHI strain alone, the *sapA::kan* mutant alone or a mixture of these two was inoculated into either the nasopharynx or the middle ears of a chinchilla (*Chinchilla lanigera*). At periodic time

points following inoculation, a nasal lavage or middle ear tapping procedure is done in order to determine the number of bacteria (in colony forming units per milliliter fluid) present in each of these anatomic sites within the uppermost airway that are extremely relevant to the disease course of otitis media.

In the competitive study wherein the parental NTHi strain and the *sapA::kan* mutant were mixed in equal parts and inoculated into chinchilla middle ears, as shown in Fig. 6, the ability of the *sapA::kan* mutant to survive in the middle ear was dramatically attenuated as compared to the parental strain. The parental strain behaved typically and was present at a very high bacterial load in the middle ears out to eight days after the challenge.

In addition, the *sapA::kan* mutant was unable to survive when inoculated in the chinchilla middle ear alone as compared to the parental strain inoculated alone. As demonstrated in Fig. 7, in both animals challenged with the *sapA::kan* mutant, the bacteria were cleared from both ears of both animals by day 19 or 27 respectively. The parental isolate continued to be culturable at high numbers from the middle ear at these time points (Fig. 7; top panel). Similarly, the *sapA::kan* mutant was unable to survive when inoculated alone into the nasopharynx of a chinchilla (Fig. 7; bottom panel). Whereas the parental isolate maintained stable colonization of the nasopharynx, the *sapA::kan* mutant was cleared 12 days after challenge.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those skilled in the art. Accordingly, only such limitations as appear in the claims should be placed on the invention